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APPENDIX D

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Expression of a Cloned P_{2Y} Purinergic Receptor that Couples to Phospholipase C

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SUMMARY

P_{2Y} purinergic receptors previously have been shown to couple either to activation of phospholipase C through a pertussis toxin-insensitive mechanism or to inhibition of adenylyl cyclase through pertussis toxin-sensitive members of the G_i family of G proteins. These and other pharmacological data strongly suggest that multiple P_{2Y} purinergic receptors exist. Webb *et al.* [*FEBS Lett.* 324:219–225 (1993)] cloned a cDNA that, when expressed in frog oocytes, displayed the general pharmacological characteristics of a P_{2Y} purinergic receptor but whose second messenger linkage was not resolved. We have now cloned the meleagrid (turkey) homologue of the previously cloned chick P_{2Y} purinergic receptor and have stably expressed it in a heterologous human cell line (1321N1 astrocytoma cells) to establish its signaling properties. The purinergic receptor agonist 2-methylthio-ATP (2MeSATP) stimulated a marked activation of phospholipase C in 1321N1 cells stably expressing the meleagrid receptor. The order of potency of a series of analogues of ATP and ADP for stimulation of phospholipase C by the receptor expressed in 1321N1 cells [2MeSATP = 2-methylthio-ADP > adenosine 5′-*O*-(2-thio)diphosphate > ADP > 2-chloro-ATP = adenosine 5′-*O*-(3-thio)triphosphate ≥ ATP > adenylyl-imidodiphosphate > UTP] was similar to that observed for P_{2Y} purinergic receptors in turkey erythrocytes and many other tissues and was markedly different from those of the P_{2U} and P_{2X} purinergic receptor subtypes. Stimulation of inositol lipid hydrolysis by P_{2Y} purinergic agonists was not affected by preincubation of cells with pertussis toxin. In contrast to its marked effects on phospholipase C activity, 2MeSATP caused only a small and variable inhibition of cAMP accumulation. Ribonuclease protection analysis of turkey tissues showed that this P_{2Y} purinergic receptor is most highly expressed in blood and brain. Taken together, these results indicate that a phospholipase-C-activating P_{2Y} purinergic receptor has been cloned and stably expressed in 1321N1 astrocytoma cells.

Extracellular adenine nucleotides interact with cell surface receptors to produce a broad range of physiological responses, and multiple receptors that recognize ATP, ADP, and synthetic analogues of these nucleotides have been described (1). These include the P_{2Y} and P_{2X} purinergic receptors, which originally were delineated in studies on smooth muscle responses, the P_{2T} purinergic receptor, which is an ADP-activated receptor on thrombocytes, the P_{2Z} purinergic receptor, which serves a non-selective pore-forming function, and the P_{2U} purinergic receptor, which is widely distributed on a variety of cell types.

Delineation of P_2 purinergic receptors has depended almost entirely on differential selectivities of analogues of ATP and ADP. 2MeSATP is a potent P_{2Y} purinergic receptor agonist, but it is not an effective agonist at P_{2X} and other purinergic receptors (2). There is strong evidence that multiple subtypes exist within several, if not all, of the classes of P_2 purinergic receptors. For example, responses to a broad range of adenine nucleotide analogues have been compared in four tissues expressing P_{2Y} purinergic receptors and three tissues expressing P_{2X} purinergic receptors (3, 4). Many of these analogues showed selectivity or specificity for certain of the P_{2Y} or P_{2X} purinergic receptor responses, suggesting that subtypes of each of these P_2 purinergic receptor types exist.

Subtypes of receptors within a given class often possess very different second messenger-coupling specificities. For example, M_1 muscarinic cholinergic and α_1-adrenergic receptors activate the inositol lipid signaling cascade through G_q family G proteins and phospholipase C, whereas M_2 muscarinic cholinergic and α_2-adrenergic receptors couple through G_i to inhibit adenylyl cyclase (5, 6). Similar contrasts can be seen for subtypes of many other G protein-linked receptors, e.g., receptors for serotonin, angiotensin, endothelin, and thromboxanes. When receptor density achieved in heterologous systems is comparable to receptor density in native tissues, selectivity in G protein/effector coupling is maintained with great fidelity for these receptor subtypes irrespective of the tissue in which they

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ABBREVIATIONS: 2MeSATP, 2-methylthio-ATP; 2ClATP, 2-chloro-ATP; 2MeSADP, 2-methylthio-ADP; α,βMeATP, α,β-methylene-ATP; β,γMeATP, β,γ-methylene-ATP; ADPβS, adenosine 5′-*O*-(2-thio)diphosphate; App(NH)p, adenylyl-imidodiphosphate; ATPγS, adenosine 5′-*O*-(3-thio)triphosphate; DMEM, Dulbecco's modified Eagle's medium; PCR, polymerase chain reaction; TCA, trichloroacetic acid; CHO, Chinese hamster ovary; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

are expressed. We have taken advantage of this property of G protein-linked receptors to provide evidence for the existence of at least two P_{2Y} purinergic receptor subtypes.

The prototypical response to P_{2Y} purinergic receptor activation in various tissues is stimulation of inositol lipid hydrolysis (7–11). However, ATP and ADP also decrease cAMP levels in various tissues (12–15), and we recently reported that the pharmacological selectivity of a series of agonists for inhibition of adenylyl cyclase in C6 glioma cells is consistent with that of a P_{2Y} purinergic receptor (16). These effects on cAMP accumulation are blocked by pertussis toxin, which indicates that coupling is through a G protein of the G_i class. Activation of this P_{2Y} purinergic receptor on C6 glioma cells has no effect on inositol lipid hydrolysis or Ca^{2+} mobilization. These results strongly support the idea that at least two P_{2Y} purinergic receptor subtypes exist, one that couples through G_i to inhibit adenylyl cyclase and another that activates phospholipase C through G proteins of the G_q family.

Unambiguous definition of receptor subtypes necessitates association of pharmacological and second messenger signaling properties with receptor proteins whose amino acid sequences have been deduced by molecular cloning. The sequences of only two P_2 purinergic receptors have been reported to date. A P_{2U} purinergic receptor, which is activated by ATP, UTP, and ATPγS, was cloned (17), and Webb *et al.* (18) have reported the sequence of a receptor cloned from chick brain cDNA that when expressed in frog oocytes confers a slowly developing, ATP-stimulated, Ca^{2+}-activated, inward current. Although limited drug concentrations were tested, this receptor displayed the general pharmacological selectivity of a P_{2Y} purinergic receptor. Whether this receptor represents a G_i/adenylyl cyclase-linked or a G_q/phospholipase C-linked receptor was not established, and therefore its relationship, if any, to the putative subtypes of P_{2Y} purinergic receptors that have been identified on the basis of second messenger coupling responses is not clear. Based on this uncertainty as well as the limitations of obtaining detailed pharmacological data in studies of receptors expressed in oocytes, we have cloned the meleagrid (turkey) homologue of the previously cloned chick P_{2Y} purinergic receptor and have stably expressed it in 1321N1 human astrocytoma cells. Activation of this receptor resulted in stimulation of phospholipase C through a pertussis toxin-insensitive pathway, and little or no effect on adenylyl cyclase was observed. Pharmacological analyses of the expressed receptor indicate that it displays drug selectivities that are remarkably similar to those previously observed for P_{2Y} purinergic receptors in a broad range of tissues.

Experimental Procedures

Materials. ATP, ADP, ADPβS, ATPγS, App(NH)p, and UTP were obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN); 2MeSATP, 2MeSADP, α,βMeATP, β,γMeATP, and 2ClATP were obtained from Research Biochemicals Inc. (Natick, MA). pcDNA3 is an expression vector developed by Invitrogen (San Diego, CA). pBS SK$^-$ is a DNA plasmid vector from Stratagene (La Jolla, CA). G418 sulfate, DMEM, and α-minimum essential medium were obtained from GIBCO/BRL (Grand Island, NY). Fetal bovine serum was obtained from Hyclone Laboratories Inc. (Logan, UT). TCA was obtained from Fisher (Fair Lawn, NJ). 1-Isobutyl-3-methylxanthine, LiCl, isoproterenol, carbachol, and apyrase type IV (EC 3.6.1.5) were obtained from Sigma Chemical Co. (St. Louis, MO). Forskolin [as the water-soluble analogue 7β-deacetyl-7β-(γ-*N*-methylpiperazino)butyryl-forskolin] was obtained from Calbiochem (La Jolla, CA). Pertussis toxin was obtained from List Biological Laboratories Inc. (Campbell, CA). Restriction enzymes were obtained from Pharmacia LKB Biotechnology (Piscataway, NJ). The Sequenase 2.0 kit was obtained from United States Biochemical (Cleveland, OH). The RPAII kit was obtained from Ambion Inc. (Austin, TX).

Cloning of turkey brain receptor. Total RNA from turkey brain was isolated by a modification of the method of Chomczynski and Sacchi (19). Briefly, frozen turkey brain (1 g of tissue) was homogenized in guanidinium thiocyanate, 2-mercaptoethanol, 0.3 M sodium acetate, pH 5.2, and extracted with acid phenol. After addition of chloroform to separate the two phases, RNA was precipitated from the aqueous phase with 2 volumes of ethanol. Total RNA (1 μg) was then reverse transcribed with dT_{17} as a primer for Moloney murine leukemia virus reverse transcriptase, using the Perkin Elmer/Cetus GeneAmp RNA kit. Two oligonucleotide primers based on the chick brain P_{2Y} purinergic receptor sequence (18) were used to amplify the turkey brain clone. The upstream primer (5′-GAGAGGATCCATCATGACCGA-AGCCCTCAT-3′) included a *Bam*HI site, the last three bases of the 5′ noncoding region, and the first 17 bases of the coding region of chick brain P_{2Y} purinergic receptor. The downstream primer (5′-TCTCTCTAGATCACAAACTGGTGTCCCCGTT-3′) included the last 18 bases of the chick brain P_{2Y} purinergic receptor coding region, a stop codon, and an *Xba*I site. The conditions for PCR amplification were 95° for 1 min and 55° for 1 min, repeated for 35 cycles. The resulting amplified cDNA was cloned into pcDNA3 and then subcloned into M13 vectors in both directions. The clones were sequenced on both strands using the Sequenase 2.0 kit and primers based on the chick brain P_{2Y} purinergic receptor sequence. The sequence of turkey brain P_{2Y} was confirmed by sequencing single clones from four separate PCR amplifications.

Transfection of mammalian cells. pcDNA3 expression vector contains a cytomegalovirus promoter for high expression levels in mammalian cells and a neomycin resistance gene for continual selection of expressing cells. Cells were transfected by the calcium phosphate precipitation method of Chen and Okayama (20). 1321N1 human astrocytoma cells were plated at a density of 1×10^6 cells/plate in 100-mm tissue culture plates and allowed to attach overnight. pcDNA3 plasmid DNA with or without receptor coding sequence was suspended in calcium phosphate buffer and incubated overnight with 1321N1 cells at 35° in 3% CO_2. The cells were washed twice with growth medium and grown for 48 hr before subculturing and selection with G418 sulfate. G418-resistant cells were subcloned by isolation and expanded, and 12–18 clones were screened for expression of the P_{2Y} purinergic receptor.

Tissue culture. 1321N1 human astrocytoma cells were grown in monolayer culture at 37° in 5% CO_2 in high-glucose DMEM supplemented with 5% fetal bovine serum. Transfected cells were maintained in medium supplemented with 900 μg/ml G418 sulfate. The growth medium was changed every fourth day and cells were subcultured at a density of 1×10^5 cells/ml of medium; postconfluent cells were used for assay on day 7.

Inositol phosphate accumulation. Inositol phosphate accumulation was determined as described previously (16), with the following exceptions. Cells were labeled overnight with 1 μCi/ml [^{3}H]inositol in inositol-free DMEM containing 5% dialyzed fetal bovine serum. Fetal bovine serum was dialyzed against 4 liters of Earle's salts changed three times over 48 hr. Earle's salts are 1.8 mM $CaCl_2$, 5.3 mM KCl, 0.8 mM $MgSO_4$, 117 mM NaCl, 26 mM $NaHCO_3$, 1 mM NaH_2PO_4, and 5.6 mM glucose, pH 7.4. Labeled cells were washed once, the medium was replaced with 890 μl of 20 mM HEPES-buffered Eagle's medium, pH 7.4, without LiCl, and the cells were maintained in a 37° water bath for 30 min before proceeding. This step was necessary to reduce background levels of [^{3}H]inositol phosphates, so that agonist-stimulated accumulation could be detected more easily. After incubation for 30 min at 37° in [^{3}H]inositol-free medium, 10 μl of LiCl were added to prelabeled cells to a final concentration of 10 mM and incubation was continued for an additional 10 min. Receptor agonists in 100 μl of medium were added, cells were incubated for 20 min at 37°, and

reactions were terminated by aspiration of the medium and addition of 1 ml of ice-cold 5% TCA. [^{3}H]Inositol phosphates were purified by anion exchange chromatography as described previously (21). [^{3}H] Phospholipids were collected after solubilization of the TCA-precipitated cells with 1 ml of 1 N NaOH.

cAMP accumulation. cAMP accumulation was determined from the conversion of [^{3}H]ATP to [^{3}H]cAMP as described previously (16), with the following exceptions. Transfected 1321N1 cells grown in 12-well plates were incubated overnight with 1 μCi/ml [^{3}H]adenine in DMEM containing 5% dialyzed fetal bovine serum. The cells were washed once with 890 μl of 20 mM HEPES-buffered Eagle's medium, pH 7.4, and incubated at 37° for 30 min before proceeding. Ten microliters of 1-isobutyl-3-methylxanthine dissolved in dimethylsulfoxide were then added to a final concentration of 200 μM and incubation was continued for an additional 10 min. Receptor agonists in 100 μl of medium were added, cells were incubated for 5 min at 37°, and the reactions were terminated by aspiration of the medium and addition of 1 ml of ice-cold 5% TCA. [^{3}H]ATP and [^{3}H]cAMP were separated by Dowex and alumina chromatography, as described previously (22).

RNase protection assays. Levels of turkey P_{2Y} purinergic receptor mRNA in various tissues were quantitated by RNase protection assay using the RPAII kit, according to the vendor's protocol. The ribonucleotide probe for the meleagrid P_{2Y} purinergic receptor was generated from a subcloned fragment that comprised nucleotides 654–927 of the coding sequence. Fragments were generated by PCR amplification using gene-specific primers and were then subcloned into pBS SK$^-$. Total RNA from turkey blood was isolated by lysis of freshly washed adult erythrocytes in guanidinium thiocyanate and sedimentation through a cesium chloride cushion as described (23). Total RNA from all other tissues was prepared by a modification of the method of Chomczynski and Sacchi (19), as described above.

Results

Isolation of a P_{2Y} purinergic receptor cDNA clone from turkey brain. We have previously studied in detail a phospholipase C-linked P_{2Y} purinergic receptor on turkey erythrocytes. The recent cloning of a P_{2Y} purinergic receptor from chick brain (18) provided the means for isolation of a turkey homologue of this receptor and allowed the second messenger coupling response and pharmacological specificity of this P_{2Y} purinergic receptor to be determined. To isolate a full length cDNA clone of the meleagrid P_{2Y} purinergic receptor, PCR amplification was performed on total RNA isolated from turkey brain, using oligonucleotide primers identical to the beginning and end of the coding sequence of the chick brain P_{2Y} purinergic receptor. The resulting amplified fragment was cloned and sequenced, and sequence analysis indicated that the meleagrid sequence was >98% identical at the nucleotide level (17 differences in 1089 nucleotides) to the chick gene.[1] The only amino acid difference between the two genes was a conservative substitution of threonine-28 to serine. Amplification of a genomic clone with the same primers resulted in a fragment identical in size to the cDNA, suggesting that the P_{2Y} purinergic receptor gene lacks an intron. Given the nearly complete identity of the two sequences, the second messenger coupling and pharmacological specificity of the meleagrid and chick homologues of the P_{2Y} purinergic receptor should be the same. Therefore, the meleagrid sequence was prepared for expression and characterization in eukaryotic cells.

Expression of meleagrid P_{2Y} purinergic receptor in 1321N1 cells and stimulation of inositol phosphate accumulation. pcDNA3 vector alone or vector containing the coding sequence of the meleagrid putative P_{2Y} purinergic receptor was transfected into 1321N1 human astrocytoma cells. These cells express no detectable endogenous receptors for adenine nucleotides (24) and endogenously express an M1 muscarinic cholinergic receptor coupled to stimulation of phospholipase C (21, 25). 1321N1 cells transfected with the P_{2Y} purinergic receptor construct (1321N1-P2Y cells) were cloned and screened for 2MeSATP-stimulated inositol phosphate accumulation or inhibition of cAMP accumulation. Initially, very little stimulation or inhibition of inositol phosphate or cAMP accumulation was observed in any of the transfected cell lines. However, certain of the 1321N1-P2Y cell clones expressed basal levels of [^{3}H]inositol phosphates that were markedly greater than basal levels in the vector-transfected clonal cell lines. We reasoned that this elevated [^{3}H]inositol phosphate accumulation could be due, at least in part, to release of endogenous ATP and/or ADP into the medium, with subsequent activation of an expressed P_{2Y} purinergic receptor and elevation of [^{3}H] inositol phosphates during the 18-hr [^{3}H]inositol labeling period. Two approaches were used in an attempt to decrease basal levels of [^{3}H]inositol phosphates. 1321N1-P2Y cells were treated with apyrase overnight during the [^{3}H]inositol labeling step, with the goal of decreasing ATP and/or ADP levels in the cell medium. Alternatively, [^{3}H]inositol-labeled 1321N1-P2Y cells were washed free of [^{3}H]inositol and incubated for 30 min in the absence of LiCl, to allow elevated levels of [^{3}H]inositol phosphates to decrease.

The data presented in Fig. 1 illustrate the results of the different treatments of pcDNA3 vector-transfected (1321N1-vector) cells and 1321N1-P2Y cells. In wild-type control (data not shown) and vector-transfected cells (Fig. 1A), 10 μM 2MeSATP and other analogues of ATP and ADP (Ref. 24 and data not shown) had no effect on [^{3}H]inositol phosphate accumulation. Activation by 500 μM carbachol of an endogenous M1 muscarinic cholinergic receptor on 1321N1 cells markedly elevated [^{3}H]inositol phosphate levels. There was a 10-fold increase in basal [^{3}H]inositol phosphate levels in cells stably transfected with the P_{2Y} purinergic receptor (Fig. 1B). A variable small increase in [^{3}H]inositol phosphate levels above the high background level occurred in response to 2MeSATP in 1321N1-P2Y cells and the large response to carbachol was maintained. If 1321N1-P2Y cells were switched to [^{3}H]inositol-free medium for 30 min before incubation with LiCl and receptor agonists (Fig. 1C), the basal level of [^{3}H]inositol phosphates was reduced, relative to 1321N1-P2Y cells that did not receive this incubation before addition of LiCl (Fig. 1, compare C and B). A marked 2MeSATP-stimulated accumulation of [^{3}H]inositol phosphates, which was approximately 50% of the level of stimulation observed with carbachol, occurred in these cells. Apyrase treatment during the overnight [^{3}H]inositol labeling step (Fig. 1D) also resulted in 1321N1-P2Y cells with reduced basal levels of radioactivity, relative to untreated 1321N1-P2Y cells (Fig. 1, compare D and B). 2MeSATP markedly stimulated inositol phosphate accumulation in apyrase-treated cells, to levels that exceeded those observed with carbachol. Therefore, when 1321N1-P2Y cells were treated with apyrase overnight or were preincubated for 30 min after a change of medium, the basal accumulation of [^{3}H]inositol phosphates was decreased and 2MeSATP-stimulated [^{3}H]inositol phosphate accumulation was readily detected. We preferred, in subsequent experiments, to decrease basal [^{3}H]inositol phosphate levels by incubating cells for 30 min after a change to [^{3}H]inositol-free

[1] The nucleotide sequence of the meleagrid P_{2Y} purinergic receptor has been submitted to the GenBank database with accession number U09842.

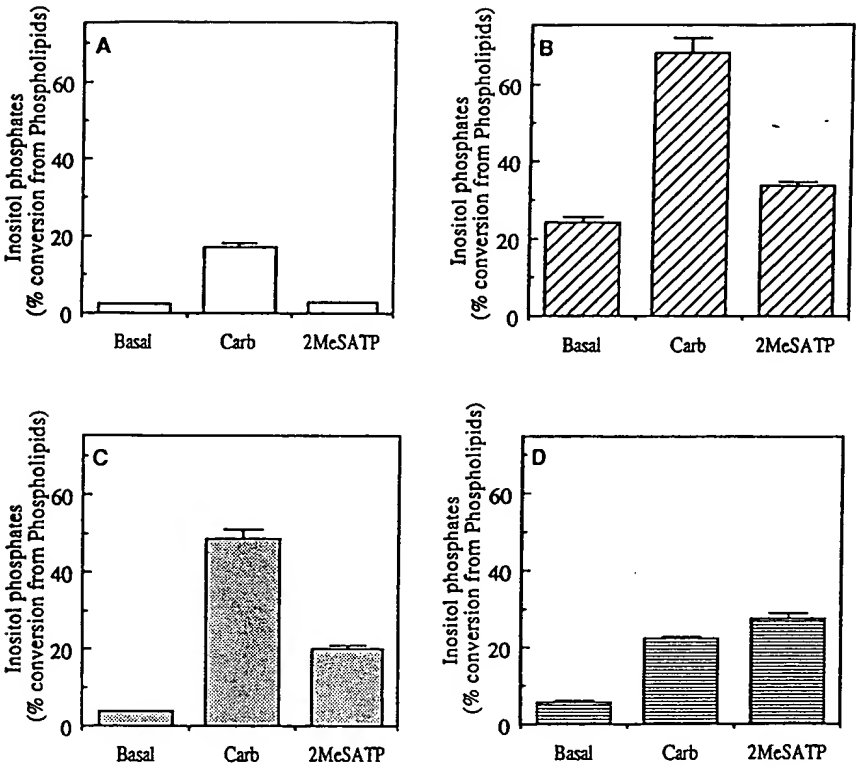

Fig. 1. Effects of preincubation and apyrase treatment on basal and agonist-stimulated levels of inositol phosphate accumulation in vector- and P_{2Y} purinergic receptor-transfected 1321N1 cells. A and B, 1321N1-vector (A) and 1321N1-P2Y (B) cells were treated overnight with [^{3}H]inositol and assayed for [^{3}H]inositol phosphate accumulation without a 30-min preincubation. C, 1321N1-P2Y cells were assayed for [^{3}H]inositol phosphate accumulation after a 30-min preincubation, as described in Experimental Procedures. D, 1321N1-P2Y cells were treated overnight, during [^{3}H]inositol labeling, with apyrase (2 units/ml) and were then assayed for [^{3}H] inositol phosphate accumulation. Data shown are mean ± standard deviation for a representative experiment assayed in quadruplicate. Similar results were obtained in two other experiments. *Carb*, carbachol.

medium instead of incubating cells overnight with apyrase, to avoid any potential hydrolytic effect of apyrase on the various adenine nucleotides used as receptor agonists. Incubation of 1321N1-vector cells overnight with apyrase or switching of these cells to [^{3}H]inositol-free medium before incubation with LiCl and agonists did not result in appearance of a 2MeSATP-stimulated response (data not shown).

Pharmacological characterization of the expressed meleagrid receptor. To characterize more fully the putative P_{2Y} purinergic receptor from turkey brain, inositol phosphate accumulation was studied with several P_{2Y}-, P_{2U}-, and P_{2X}-selective and nonselective agonists. As anticipated from the almost complete sequence identity with the previously cloned chick P_{2Y} purinergic receptor, the meleagrid receptor showed the pharmacological profile expected of a P_{2Y} purinergic receptor (Fig. 2; Table 1). Adenine nucleotides previously shown to be effective P_{2Y} purinergic receptor agonists were all full agonists at the expressed receptor, with little variation in the maximal response obtained within an experiment. 2MeSATP and 2MeSADP, which are agonists that were previously shown to be selective for P_{2Y} purinergic receptors (2, 3, 10, 16), showed the highest potency for stimulation of inositol phosphate accumulation in 1321N1-P2Y cells. The P_{2X} purinergic receptor-selective agonists α,βMeATP and β,γMeATP did not stimulate inositol phosphate accumulation in 1321N1-P2Y cells. Furthermore, UTP had little effect, indicating that the expressed receptor is not a P_{2U} purinergic receptor subtype. The pharmacological profile obtained for stimulation of inositol phosphate accumulation in 1321N1-P2Y cells [2MeSATP = 2MeSADP > ADPβS > ADP > 2ClATP = ATPγS ≥ ATP > App(NH)p > UTP] was very similar to that obtained for the P_{2Y} purinergic receptor coupled to inhibition of adenylyl cyclase activity in rat C6–2B glioma cells (Table 1). With the exception of the potencies of ADP and 2ClATP, the pharmacological profile of the expressed receptor was similar to that previously

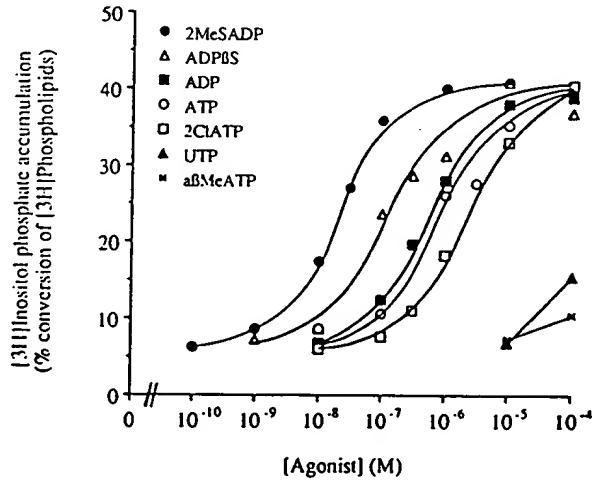

Fig. 2. Concentration-effect curves for purinergic receptor agonists in P_{2Y} purinergic receptor-transfected 1321N1 cells. Inositol phosphate accumulation was measured in [^{3}H]inositol-prelabeled 1321N1-P2Y cells that had been subjected to a 30-min preincubation in [^{3}H]inositol-free medium, as described in Experimental Procedures. Concentration-effect curves were generated by treating cells for 20 min with varying concentrations of agonist. *Curves* represent mean data obtained from a single experiment assayed in quadruplicate. Similar results were obtained in two or three other experiments.

obtained for P_{2Y} purinergic receptors coupled to phospholipase C in turkey erythrocyte membranes (4, 10).

P_{2Y} purinergic receptors previously were shown to couple through a pertussis toxin-insensitive mechanism to activation of phospholipase C or through pertussis toxin-sensitive members of the G_i family of G proteins to inhibition of adenylyl cyclase. Stimulation by either 2MeSATP or carbachol of inositol phosphate accumulation in 1321N1-P2Y cells was not sensitive to pretreatment of cells with 100 ng/ml pertussis toxin (Table 2). This concentration of pertussis toxin previ-

TABLE 1

Relative potencies of purinergic receptor agonists for activation of P_{2Y} purinergic receptors

Inositol phosphate accumulation was measured in 1321N1-P2Y cells as described in Experimental Procedures. Concentration-effect curves were generated by incubating [^{3}H]inositol-labeled cells for 20 min with varying concentrations of agonist, as illustrated in Fig. 2. $K_{0.5}$ values were calculated as the concentration of agonist required to produce a half-maximal effect, as determined by nonlinear regression analysis. Data shown are mean ± standard error of $K_{0.5}$ values for three or four independent experiments.

Agonist	$K_{0.5}$		
	1321N1-P2Y	C6-2B[a]	Turkey erythrocyte[b]
		nM	
2MeSATP	30.5 ± 14.8	3.9 ± 1.3	8 ± 2
2MeSADP	25.7 ± 9.6	10.0 ± 5.3	6 ± 3
ADPβS	233 ± 76	675 ± 118	96 ± 27
ADP	534 ± 61	3,000 ± 580	8,000 ± 2,000
ATPγS	2,790 ± 330	3,166 ± 1,014	1,260 ± 380
2ClATP	2,990 ± 1,320	2,140 ± 761	72 ± 19
ATP	4,120 ± 1,970	9,000 ± 1,703	2,800 ± 700
App(NH)p	>10,000	>10,000	4,450 ± 1,150
UTP	>10,000	>10,000	143,000 ± 44,000
αβMeATP	NE[c]	NE	>100,000
βγMeATP	NE	NE	>100,000

[a] Values for P_{2Y} receptor-promoted inhibition of cAMP accumulation in rat C6-2B glioma cells, obtained from Boyer *et al.* (16).

[b] Values for P_{2Y} receptor-mediated activation of phospholipase C in turkey erythrocyte membranes, obtained from Burnstock *et al.* (4)

[c] NE, no effect at 100 μM.

TABLE 2

Effect of pertussis toxin treatment on receptor-stimulated inositol phosphate accumulation in 1321N1-P2Y cells

1321N1-P2Y cells were treated for 18 hr with vehicle (control) or with 100 ng/ml pertussis toxin. The cells were washed, preincubated for 30 min in [^{3}H]inositol-free medium, and assayed for inositol phosphate accumulation in the absence of added drug (basal) or in the presence of 500 μM carbachol or 10 μM 2MeSATP. [^{3}H] Inositol phosphate accumulation was quantitated as described in Experimental Procedures, and the data are mean ± standard error for three experiments assayed in quadruplicate.

	[^{3}H]Inositol phosphate accumulation		
	Basal	Carbachol	2MeSATP
	% conversion		
Control	16.1 ± 1.3	66.6 ± 15.0	49.4 ± 7.0
Pertussis toxin	17.1 ± 3.3	69.7 ± 17.6	51.0 ± 5.8

ously was shown to cause full ADP-ribosylation of pertussis toxin-sensitive G proteins in 1321N1 cells and to completely block G_i-mediated inhibition of adenylyl cyclase (26, 27).

In addition to activation of phospholipase C, P_{2Y} purinergic receptors previously were shown to inhibit adenylyl cyclase. Our data with C6 glioma cells suggest that these two different second messenger responses occur through activation of two different P_{2Y} purinergic receptor subtypes (16). However, this remains to be formally proven. Thus, it was important to determine whether the cloned P_{2Y} purinergic receptor coupled to adenylyl cyclase in addition to phospholipase C. cAMP levels were elevated by isoproterenol-mediated stimulation of the β-adrenergic receptors endogenously expressed on 1321N1 cells. Incubation of 1321N1-P2Y cells with isoproterenol plus 2MeSATP resulted in a slight increase in cAMP accumulation over that observed with isoproterenol alone (Fig. 3). This increase may be secondary to inositol-1,4,5-trisphosphate-promoted Ca^{2+} mobilization and Ca^{2+}-mediated stimulation of adenylyl cyclase activity. Similar results were previously observed during stimulation of 1321N1 cells with carbachol (28, 29). To fully activate adenylyl cyclase activity, 1321N1-P2Y cells were incubated with isoproterenol plus forskolin. Data

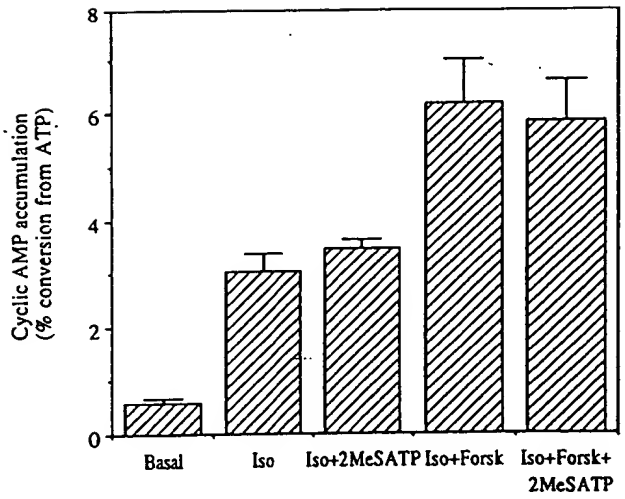

Fig. 3. Absence of 2MeSATP-promoted inhibition of cAMP accumulation in P_{2Y} receptor-transfected 1321N1 cells. 1321N1-P2Y cells were prelabeled with [^{3}H]adenine overnight and subjected to a 30-min incubation in [^{3}H]adenine-free medium, as described in Experimental Procedures. The labeled and washed cells were then incubated for 5 min in the absence of added drug (*Basal*) or in the presence of 10 μM isoproterenol (*Iso*), 10 μM isoproterenol plus 10 μM 2MeSATP (*Iso+2MeSATP*), 10 μM isoproterenol plus 10 μM forskolin (*Iso+Forsk*), or 10 μM isoproterenol plus 10 μM forskolin plus 10 μM 2MeSATP (*Iso+Forsk+2MeSATP*) and were assayed for cAMP accumulation as described in Experimental Procedures. The data shown are mean ± standard error for three to nine experiments assayed in quadruplicate.

averaged from nine experiments showed that 2MeSATP had no effect on isoproterenol- plus forskolin-stimulated cAMP accumulation (Fig. 3). However, in four of the nine experiments a small (20–30%) but significant inhibition of cAMP accumulation occurred. This finding suggests that, whereas stimulation of inositol phosphate accumulation is the primary signaling pathway for this P_{2Y} purinergic receptor, some coupling to inhibition of adenylyl cyclase activity may occur. Desensitization of P_{2Y} purinergic receptors is not responsible for the lack of inhibition, because incubation of 1321N1-P2Y cells overnight with 2 units/ml apyrase before assay did not reveal any inhibition by 2MeSATP of cAMP accumulation (data not shown).

Tissue distribution of the cloned meleagrid P_{2Y} purinergic receptor. RNase protection assays on total RNA isolated from various meleagrid tissues were performed to establish the localization of P_{2Y} purinergic receptor transcripts. An antisense ribonucleotide probe specific for the P_{2Y} purinergic receptor sequence was constructed, and this probe hybridized most strongly with RNA from turkey brain, lung, and blood (Fig. 4). Lesser intensity hybridization was observed with RNA from stomach, gut, and skeletal muscle. With the exception of hybridization levels in lung, these results correlate with data obtained by Webb *et al.* (18) by Northern blot analysis of chick tissues.

Discussion

The relative potencies of a series of 11 adenine nucleotide analogues for stimulation of inositol phosphate accumulation confirm that the meleagrid receptor that has been stably expressed in 1321N1 human astrocytoma cells is a P_{2Y} purinergic receptor (Fig. 2; Table 1). Previous comparative studies of agonist potencies across a number of tissues have led to the suggestion that multiple subtypes of P_{2Y} purinergic receptors

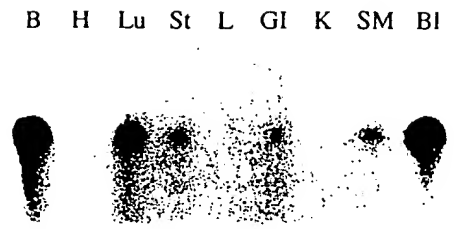

Fig. 4. Tissue distribution of the cloned meleagrid P_{2Y} purinergic receptor. RNase protection assays were performed on 25 μg of total RNA isolated from various meleagrid tissues, using a 274-base pair antisense ribonucleotide probe specific for the cloned meleagrid P_{2Y} purinergic receptor, as described in Experimental Procedures. *B*, brain; *H*, heart; *Lu*, lung; *St*, stomach; *L*, liver; *GI*, gastrointestinal tract; *K*, kidney; *SM*, skeletal muscle; *Bl*, blood.

exist (3, 4). No firm subclassification has been established on the basis of these tissue responses, and it is not yet possible to unambiguously associate the receptor that has been cloned and expressed here with any given set of tissue responses. However, within the limitations of the agonists that have been studied, this P_{2Y} purinergic receptor expresses pharmacological specificities not unlike those of the P_{2Y} purinergic receptor of the guinea pig taenia coli, rat C6 glioma cells, and turkey erythrocytes.

We believe that studies on second messenger responses may more strongly suggest the existence of subtypes of P_{2Y} purinergic receptors than do the differences in agonist potencies that have been observed in tissue responses. Thus, P_{2Y} purinergic receptors have been shown to activate phospholipase C and inhibit adenylyl cyclase. In two model systems that have been studied in detail, the P_{2Y} purinergic receptor couples either to G_i and adenylyl cyclase (16) or to G_{11} and phospholipase C (10), but not to both. Although this does not prove that a similar fidelity of coupling will be observed in all tissues, this is a strong possibility, based on the strict selectivity of coupling to different G proteins and second messenger cascades that has been observed for subtypes in many receptor classes. Expression of the meleagrid receptor in 1321N1 human astrocytoma cells conferred marked responsiveness of phospholipase C to activation by P_{2Y} purinergic receptor agonists. Activation of phospholipase C was completely insensitive to pertussis toxin. These results are consistent with the idea that a P_{2Y} purinergic receptor subtype that couples through a G_q type of G protein to activate phospholipase C has been cloned. In preliminary experiments with CHO cells that were stably transfected with the meleagrid P_{2Y} purinergic receptor, we have observed a similar marked stimulation by P_{2Y} purinergic receptor agonists of inositol phosphate accumulation.[2] This suggests that linkage to phospholipase C is an intrinsic property of this P_{2Y} purinergic receptor subtype and not of the cell line.

A small variable effect of 2MeSATP on the inhibition of cAMP accumulation in transfected 1321N1-P2Y cells was observed in four of nine experiments. Overexpression of the receptor protein in 1321N1 cells may allow the receptor to couple weakly to other G protein subunits and thus produce the variable inhibition of adenylyl cyclase activity that was seen. Such a result was originally observed with overexpression of transfected muscarinic receptors in CHO cells, in which a G_i-coupled m2 muscarinic receptor was found to couple weakly to phospholipase C (30). The physiological importance of such secondary coupling is not known, but the coupling is presumed to be an artifact of the presence of unnaturally high levels of receptor protein. An important next target for molecular cloning will be the G_i-linked P_{2Y} purinergic receptor that exclusively inhibits adenylyl cyclase in C6 glioma cells and in other tissues.

This laboratory has extensively studied a P_{2Y} purinergic receptor linked to activation of phospholipase C on turkey erythrocytes (10, 11, 31–33). Because we have no protein sequence for this receptor, we cannot with certainty equate the meleagrid P_{2Y} purinergic receptor that has been cloned with the erythrocyte signaling protein. However, RNase protection experiments indicate that the meleagrid P_{2Y} purinergic receptor mRNA is found at highest levels in blood and brain. The activation of phospholipase C observed with the receptor expressed in 1321N1 cells is very consistent with the signaling activity of the erythrocyte receptor, and the overall potencies of agonists also closely match. The differences observed in agonist (e.g., 2ClATP and ADP) potencies between the turkey erythrocyte P_{2Y} purinergic receptor and the transfected P_{2Y} purinergic receptor may be due to differences in assay conditions, because phospholipase C activity was measured in erythrocyte membranes rather than in intact cells. Exploration of these differences awaits a means for more direct comparison of the expressed and endogenous P_{2Y} purinergic receptors.

We previously used [^{35}S]ADPβS to label P_{2Y} purinergic receptors on plasma membranes purified from turkey erythrocytes (31). Although a complete understanding has not been obtained, extensive analysis of the turkey plasma membrane [^{35}S]ADPβS binding site with a very broad range of analogues of ATP and ADP has led us to question the validity of the conditions previously reported for putative P_{2Y} purinergic receptor labeling.[3] These caveats notwithstanding, [^{35}S]ADPβS was used in preliminary experiments in an attempt to label P_{2Y} purinergic receptors in transfected cells. No reproducible difference in total [^{35}S]ADPβS binding was observed between control cells and cells expressing the P_{2Y} purinergic receptor construct. This lack of a difference in radioligand binding was observed in both intact cell and membrane binding assays. Because [^{35}S]ADPβS binding was not inhibited by 2MeSATP in either control or P_{2Y} purinergic receptor-transfected cells, no specific binding of the radiolabeled agonist could be detected. The availability of a radiolabeled, high affinity, P_{2Y} purinergic receptor antagonist may be necessary to quantitate P_{2Y} purinergic receptor levels in cells stably expressing this receptor.

The observations described here have several implications in the study of P_2 purinergic receptors. The experiments with apyrase suggest, but do not prove, that release of ATP/ADP occurs from 1321N1 human astrocytoma cells and that this released nucleotide activates the expressed P_{2Y} purinergic receptors. A similar phenomenon has been observed during expression of human P_{2U} purinergic receptors in the same cells (34). The difference in response to 2MeSATP versus carbachol in apyrase-treated cells, compared with the difference in cells not receiving apyrase treatment (Fig. 1, compare D with B and C), suggests that considerable down-regulation of P_{2Y} purinergic receptors may occur as a consequence of released ATP/ADP. Thus, the level of expression of a P_2 purinergic receptor in a given cell likely depends on the extent to which that cell releases adenine nucleotides. A corollary to this could be that the best choice for expression of a P_{2Y} purinergic receptor might be a cell type, e.g., an epithelial cell line such as HT-29 human

[2] T. M. Filtz and T. K. Harden, unpublished observations.

[3] T. K. Harden, unpublished observations.

colon carcinoma cells, that expresses a very active endogenous P_{2U} purinergic receptor (35), or even rat C6 glioma cells, which express a P_{2Y} purinergic receptor that does not activate phospholipase C but, rather, inhibits adenylyl cyclase (16). The possibility also exists that cells not apparently expressing a P_{2Y} or P_{2U} purinergic receptor under normal culture conditions may nevertheless do so if conditions are changed to minimize the effect of released ATP/ADP. For example, an endogenous P_2 purinergic receptor-activated phospholipase C response was revealed in CHO cells that were treated overnight with apyrase.[4]

The meleagrid P_{2Y} purinergic receptor is remarkably similar to the previously cloned chick P_{2Y} purinergic receptor. The single amino acid difference is a conservative substitution that retains a putative glycosylation site near the amino terminus. This conservation of sequence would suggest that the chick homologue is also a phospholipase C-linked purinergic receptor. Purinergic receptors should share characteristics with other members of the G protein-linked receptor superfamily of proteins, including the existence of multiple subtypes linked to different second messenger systems. We believe that additional subtypes of P_{2Y} purinergic receptors, including those linked to inhibition of adenylyl cyclase activity, await isolation and sequencing. A comparison of tissues exhibiting P_{2Y} purinergic receptor-mediated responses (1) with the tissue distribution of transcripts for the cloned P_{2Y} purinergic receptor (Fig. 4 and Ref. 18) reveals a curious lack of P_{2Y} purinergic receptor mRNA in heart. Such results could be suggestive of the presence of another subtype of P_{2Y} purinergic receptor in cardiac tissue.

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References

1. Dubyak, G. R., and C. El-Moatassim. Signal transduction via P_2 purinergic receptors for extracellular ATP and other nucleotides. *Am. J. Physiol.* 34:C577–C606 (1993).
2. Burnstock, G., and C. Kennedy. Is there a basis for distinguishing two types of P_2 purinoceptor? *Gen. Pharmacol.* 16:433–439 (1985).
3. Fischer, B., J. L. Boyer, C. H. V. Hoyle, A. U. Ziganshin, A. L. Brizzolara, G. E. Knight, J. Zimmet, G. Burnstock, T. K. Harden, and K. A. Jacobson. Identification of potent, selective P_{2Y} purinoceptor agonists: structure-activity relationships for 2-thioether derivatives of adenosine-5′-triphosphate. *J. Med. Chem.* 36:3937–3946 (1993).
4. Burnstock, G., B. Fischer, C. H. V. Hoyle, M. Maillard, A. U. Ziganshin, A. L. Brizzolara, A. von Isakovics, J. L. Boyer, T. K. Harden, and K. A. Jacobson. Structure-activity relationships for derivatives of adenosine 5′-triphosphate as agonists at P_2 purinoceptors: heterogeneity within P_{2X} and P_{2Y} subtypes. *Drug Dev. Res.* 31:206–219 (1994).
5. Lechleiter, J., E. Peralta, and D. Clapham. Diverse functions of muscarinic acetylcholine receptor subtypes. *Trends Pharmacol Sci.* (suppl.) 34–38 (1989).
6. Insel, P. A. Structure and function of *alpha*-adrenergic receptors. *Am. J. Med.* 87:12S–18S (1989).
7. Charest, R., P. F. Blackmore, and J. H. Exton. Characterization of responses of isolated rat hepatocytes to ATP and ADP. *J. Biol. Chem.* 260:15789–15794 (1985).
8. Pirotton, S., E. Raspe, D. Demolle, C. Erneux, and J.-M. Boeynaems. Involvement of inositol 1,4,5-trisphosphate and calcium in the action of adenine nucleotides on aortic endothelial cells. *J. Biol. Chem.* 262:17461–17466 (1987).
9. Forsberg, E. J., G. Feuerstein, E. Shohami, and H. B. Pollard. Adenosine triphosphate stimulates inositol phospholipid metabolism and prostacyclin formation in adrenal medullary endothelial cells by means of P_2 purinergic receptors. *Proc. Natl. Acad. Sci. USA* 84:5630–5634 (1987).
10. Boyer, J. L., C. P. Downes, and T. K. Harden. Kinetics of activation of phospholipase C by P_{2Y} purinergic receptor agonists and guanine nucleotides. *J. Biol. Chem.* 264:884–890 (1989).
11. Berrie, C. P., P. T. Hawkins, L. R. Stephens, T. K. Harden, and C. P. Downes. Phosphatidylinositol 4,5-bisphosphate hydrolysis in turkey erythrocytes is regulated by P_{2Y} purinoceptors. *Mol. Pharmacol.* 35:526–532 (1989).
12. Okajima, F., Y. Tokumitsu, Y. Kondo, and M. Ui. P_2 purinergic receptors are coupled to two signal transduction systems leading to inhibition of cAMP generation and to production of inositol triphosphate in rat hepatocytes. *J. Biol. Chem.* 262:13483–13490 (1987).
13. Pianet, I., M. Merle, and J. Labouesse. ADP and, indirectly, ATP are potent inhibitors of cAMP production in intact isoproterenol-stimulated C6 glioma cells. *Biochem. Biophys. Res. Commun.* 163:1150–1157 (1989).
14. Valeins, H., M. Merle, and J. Labouesse. Pre-steady state study of the β-adrenergic and purinergic receptor interaction in C6 cell membranes: undelayed balance between positive and negative coupling to adenylyl cyclase. *Mol. Pharmacol.* 42:1033–1041 (1992).
15. Yamada, M., Y. Hamamori, H. Akita, and M. Yokoyama. P_2 purinoceptor activation stimulates phosphoinositide hydrolysis and inhibits accumulation of cAMP in cultured ventricular myocytes. *Circ. Res.* 70:477–485 (1992).
16. Boyer, J. L., E. R. Lazarowski, X.-H. Chen, and T. K. Harden. Identification of a P_{2Y} purinergic receptor that inhibits adenylyl cyclase. *J. Pharmacol. Exp. Ther.* 267:1140–1146 (1993).
17. Lustig, K. D., A. K. Shiau, A. J. Brake, and D. Julius. Expression cloning of an ATP receptor from mouse neuroblastoma cells. *Proc. Natl. Acad. Sci. USA* 90:5113–5117 (1993).
18. Webb, T. E., J. Simon, B. J. Krishek, A. N. Bateson, T. G. Smart, B. F. King, G. Burnstock, and E. A. Barnard. Cloning and functional expression of a brain G protein-coupled ATP receptor. *FEBS Lett.* 324:219–225 (1993).
19. Chomczynski, P., and N. Sacchi. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* 162:156–159 (1987).
20. Chen, C., and H. Okayama. High efficiency transformation of mammalian cells by plasmid DNA. *Mol. Cell. Biol.* 7:2745–2752 (1987).
21. Nakahata, N., and T. K. Harden. Regulation of inositol trisphosphate accumulation by muscarinic cholinergic and H_1-histamine receptors in human astrocytoma cells: differential induction of desensitization by agonists. *Biochem. J.* 241:337–344 (1987).
22. Harden, T. K., A. G. Scheer, and M. M. Smith. Differential modification of the interaction of cardiac muscarinic cholinergic and β-adrenergic receptors with a guanine nucleotide binding site(s). *Mol. Pharmacol.* 21:570–580 (1982).
23. Sambrook, J., E. F. Fritsch, and T. Maniatis. *Molecular Cloning: A Laboratory Manual.* Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1989).
24. Brown, H. A. Identification and characterization of a novel 5′-nucleotide receptor coupled to phospholipase C through a guanine nucleotide regulatory binding protein(s) in human airway epithelial cells. Ph.D. thesis, University of North Carolina, Chapel Hill, NC (1992).
25. Masters, S. B., T. K. Harden, and J. H. Brown. Relationships between phosphoinositide and Ca^{2+} responses to muscarinic agonists in astrocytoma cells. *Mol. Pharmacol.* 26:149–155 (1984).
26. Hughes, A. R., M. W. Martin, and T. K. Harden. Pertussis toxin differentiates between two mechanisms of attenuation of cyclic AMP accumulation by muscarinic cholinergic receptors. *Proc. Natl. Acad. Sci. USA* 81:5680–5684 (1984).
27. Evans, T., M. W. Martin, A. R. Hughes, and T. K. Harden. Guanine nucleotide-sensitive, high affinity binding of carbachol to muscarinic cholinergic receptors of 1321N1 astrocytoma cells is insensitive to pertussis toxin. *Mol. Pharmacol.* 27:32–37 (1985).
28. Meeker, R. B., and T. K. Harden. Muscarinic cholinergic receptor-mediated activation of phosphodiesterase. *Mol. Pharmacol.* 22:310–319 (1982).
29. Meeker, R. B., and T. K. Harden. Muscarinic cholinergic receptor-mediated control of cyclic AMP metabolism. *Mol. Pharmacol.* 23:384–392 (1983).
30. Ashkenazi, A., J. W. Winslow, E. G. Peralta, G. L. Peterson, M. I. Schimerlik, D. J. Capon, and J. Ramachandran. An M2 muscarinic receptor subtype coupled to both adenylyl cyclase and phosphoinositide turnover. *Science (Washington D. C.)* 238:672–675 (1987).
31. Cooper, C. L., A. J. Morris, and T. K. Harden. Guanine nucleotide-sensitive interaction of a radiolabelled agonist with a phospholipase C-linked P_{2Y} purinergic receptor. *J. Biol. Chem.* 264:6202–6206 (1989).
32. Boyer, J. L., and T. K. Harden. Irreversible activation of phospholipase C-coupled P_{2Y} purinergic receptors by 3′-*O*-(4-benzoyl)benzoyl adenosine 5′-triphosphate. *Mol. Pharmacol.* 36:831–835 (1989).
33. Boyer, J. L., C. L. Cooper, and T. K. Harden. [^{32}P]3′-*O*-(4-benzoyl)benzoyl ATP as a photoaffinity label for a phospholipase C-coupled P_{2Y} purinergic receptor. *J. Biol. Chem.* 265:13515–13520 (1990).
34. Parr, C. E., D. M. Sullivan, A. M. Paradiso, E. R. Lazarowski, L. H. Burch, J. C. Olsen, L. Erb, G. A. Weisman, R. C. Boucher, and J. T. Turner. Cloning and expression of a human P_{2U} nucleotide receptor, a target for cystic fibrosis pharmacology. *Proc. Natl. Acad. Sci. USA*, 91:3275–3279 (1994).
35. Lazarowski, E. R., and T. K. Harden. Identification of a uridine nucleotide-selective G protein-linked receptor that activates phospholipase C. *J. Biol. Chem.* 269:11830–11836 (1994).

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[4] T. M. Filtz and T. K. Harden, unpublished observations.